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Virulence and Attenuation of Murine Cytomegalovirus

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Murine cytomegalovirus (MCMV) was rapidly and regularly attenuated by passage through mouse embryo cell culture. This attenuation was manifested by alteration of lethality for suckling mice and by a striking loss of capacity to multiply in liver and spleen of weanling mice. The attenuation was selective in that the passaged virus multiplied vigorously in other organs and established high titer infections in submaxillary glands and pancreas comparable to those seen with wild virus. Furthermore, attenuated virus no longer induced transient suppression of antibody and interferon responsiveness which was a regular feature of wild MCMV infection. Wild and attenuated MCMV shared the property of being poor immunogens. They induced anti-CMV complement-fixing or neutralizing antibody very slowly with barely detectable levels present at the end of the first 2 weeks of infection. The close antigenic relationship between wild and attenuated agents was demonstrated by nearly identical neutralization by a rabbit antiserum induced with wild MCMV. Furthermore, survivors of neonatal infection with attenuated virus were fully protected against subsequent challenge with potentially lethal doses of wild MCMV. Virulence could be rapidly restored by back passage in mice.

Murine cytomegalovirus (MCMV) is virulent for immature mice. Relatively low doses of virus exert rapidly lethal effects on newborn animals. As the host matures, increasingly high concentrations of viral inoculum are necessary to produce overt disease and death (6). However, in older mice doses that result in negligible mortality or morbidity induce temporary alterations of host response capabilities which include suppression of both antibody (8) and interferon responses (9) and permissiveness to at least one infectious agent [Newcastle disease virus (NDV)] which could not otherwise multiply in a murine host (10). The mechanisms of these several biological effects of MCMV are as yet unclear.

These investigations were prompted by the initial observation that serial passage of MCMV in mouse embryo cells in culture resulted in rapid alteration of several features of its virulence for both neonatal and older animals. It was felt that a close examination of this attenuation phenomenon might shed further light on the pathogenesis of virulent (wild) MCMV in mice. Studies reported here explored the various features of this attenuation including its rapidity, durability, and the extent to which different parameters of MCMV infection were affected.

MATERIALS AND METHODS

Mice. These studies involved two randomly outbred strains of mice: CD-1 Swiss white mice obtained from Charles River Farms (Arlington, Mass.) and HA-ICR mice from A. R. Schmidt (Madison, Wis.). Major findings were confirmed in each strain. Since no detectable differences in results were found, data will be reported without reference to strain.

Viruses. Murine cytomegalovirus was of the Smith strain (9) and had been maintained by continuous mouse to mouse passage by either the intraperitoneal (ip) or subcutaneous routes for over 100 passages. Pools of MCMV designated $MCMV_0$ or "wild" in these studies were prepared by harvesting submaxillary glands 3 weeks after ip inoculation of weanling mice with $10^{4.7}$ plaque-forming units (PFU) of continuously mouse-passaged MCMV. The salivary glands were ground with mortar and pestle to a 10% suspension in equal parts of 50% sorbitol and medium 199 with 5% calf serum and 0.21% bicarbonate, or in some cases medium 199 with 10% dimethyl sulfoxide was used in place of sorbitol as a stabilizer. Such pools were then stored at −70 C in vials. Individual vials were discarded after a single use.

Pools of tissue culture-passaged MCMV were prepared by centrifugal inoculation (11) of either wild or passaged material at a 10^{-1} dilution (MOI 0.1 to 1.0) in secondary mouse embryo cell cultures (MECC) followed by harvest of tissue culture fluid when cell monolayers showed a cytopathic effect (CPE) of 50 to 75%. Such fluid was then clarified by light centrifuga-

tion, mixed with an equal volume of 50% sorbitol, and stored in the usual manner. Each pool was designated with a passage number denoting the number of times the agent had been in cell culture without intervening mouse passage. Thus, $MCMV_5$ was that virus pool harvested from the fifth serial passage in mouse embryo cells.

Two separate series of passages, one from each of two different pools of wild MCMV, were made to ensure that findings reported here were not the chance results of a random event. There was no significant difference in data obtained with each of the two passage series. Most studies concerned the effects of the first, third, and fifth passage virus pools ($MCMV_1$, $MCMV_3$, and $MCMV_5$) prepared in one passage series.

Clones of MCMV were prepared by two successive end-dilution selections of plaques under starch with neutral red stain. Isolated microplaques were aspirated with a Pasteur pipette, inoculated into MECC monolayers, and fluids were harvested when cultures reached a CPE of 50 to 90%. Of the six clones studied, one large plaque-former and five plaques of representative size were included. No differences between clones were ascertained, and these distinctions will thus be omitted.

Newcastle disease virus (NDV) was prepared in 10-day embryonated hen's eggs and assayed in chick embryo cell culture as previously described (10); the CG-9 strain was used.

Encephalomyocarditis virus (EMC) was grown and assayed in L cells and used as clarified tissue culture fluid stored at −70 C.

Cell cultures. Mouse embryo cell cultures were prepared as previously described (9) and were used for all experiments involving passage or assay of MCMV. Secondary cultures were grown in 1-oz prescription bottles to facilitate centrifugal adsorption of MCMV inocula.

An interferon-sensitive line of mouse L cells was kindly supplied by Julius Youngner and was used in most experiments for assay of interferon. It was found that MECC and these L cells yielded nearly identical interferon titers in plaque-reduction assays, and thus data from experiments with either type of cell were considered comparable to that reported previously.

Media and reagents. Growth and maintenance media for MECC consisted of medium 199, bicarbonate, antibiotics, and 10% of 5% calf serum, respectively. Lactalbumin hydrolysate (0.25%) was added to the growth medium. L cells were grown in minimal essential medium or medium 199 with 10% calf serum and 0.10% bicarbonate in 32-oz prescription bottles. For use in experiments they were trypsinized, dispensed in 25-cm^2 flasks (Falcon Plastic, Div. of B-D Laboratories, Inc., Los Angeles, Calif.), and grown to confluency in 24 to 48 hr.

In preliminary experiments tragacanth overlays (7) were found to yield results identical with those obtained with overlays incorporating lyophilized potato starch, and because of easier handling and removal tragacanth was used in all plaque assays. It was prepared as a twice-concentrated suspension in water, autoclaved, and stored at 4 C until use. It was then mixed with appropriate quantities of water, 5 parts of medium 199, 7.5% bicarbonate, and antibiotics to obtain a final tragacanth concentration of 0.8 g/100 ml and used in place of a semisolid overlay. Experiments were terminated by decanting the tragacanth overlay and washing monolayers thoroughly with buffered saline. Monolayers were then fixed and stained with crystal violet.

Sheep erythrocytes (SRBC) were washed at least three times with buffered saline prior to enumeration and dilution. Final concentrations were 2 to 4×10^8 SRBC/0.2 ml in all experiments involving SRBC as antigen in mice. In complement-fixation studies sensitized SRBC were prepared as a 2% suspension and incubated with an equal volume of Bacto anti-sheep hemolysin (1:2,000 dilution, calibrated as 2 units) for 15 min prior to use.

Complement was obtained as fresh, frozen guinea pig serum and used at a dilution of 1:100 for serum anti-SRBC hemolysin assays and at a 1:70 dilution (measured to represent 2 units) in complement-fixation studies.

Anti-MCMV serum was prepared in a domestic rabbit by initial intramuscular and subcutaneous injections, spaced 2 weeks apart, of $MCMV_0$ in complete Freund's adjuvant. After two further $MCMV_0$ inoculations ip without adjuvant over a 3 month interval, the animals was bled by cardiac puncture. Serum was heat-inactivated at 56 C for 30 min and stored at −20 C.

Methods of MCMV assay. Earlier studies concerning MCMV pathogenesis have reported quantities of infectious virus in terms of standard tube dilution or plaque assay. Subsequently, it was found that centrifugal adsorption of MCMV onto secondary MECC monolayers greatly enhanced the quantity of measurable virus (11). In this study inocula are reported again in terms of standard assay to allow comparison with previous reports, unless otherwise noted. Values for centrifugal assay of identical material were usually about 30-fold greater. However, assay of tissues for MCMV when replication of attenuated virus was under study was done entirely by centrifugal adsorption in order to maximize titers of identifiable infectious virus.

Methods of serum analysis. Mouse sera were obtained by either retroorbital or axillary exsanguination. Interferon assay of mouse sera was performed as previously described (9) except that interferon-sensitive L cells and EMC challenge virus were used in the plaque-reduction assay.

Sera were analyzed for hemagglutinin and hemolysin in microtiter plates. Serial twofold dilutions of serum were made in 0.025 ml of buffered saline and 1% fresh frozen guinea pig serum was added. The assays were read after 30 min of incubation at room temperature.

In plaque-reduction neutralization assays of mouse sera for neutralizing antibody, sera were incubated with 80 to 160 PFU of wild MCMV at 37 C for 1 hr with intermittent mixing and then plated in duplicate on MECC monolayers grown in 1-oz prescription bottles. Virus was then adsorbed by stationary incubation for 90 min at 37 C after which bottles were de-

canted and tragacanth overlay was added. MCMV plaques were enumerated in these and all other experiments under 10× magnification after at least 96 hr of further incubation at 37 C.

Complement-fixing (CF) antibody was detected by microcomplement fixation methods. Standard CF antigen was prepared from the livers of adult mice inoculated 4 days previously with 10^6 PFU of MCMV. The livers were suspended to 10% in 0.066 M tris-(hydroxymethyl) aminomethane (Tris) buffer (*p*H 7.5), clarified by light centrifugation, and stored at 4 C. Serum-antigen mixtures were incubated with 2 units of complement for 18 hr at 4 C after which 1% sensitized SRBC were added. The mixtures were then incubated for 1 hr at 37 C, and end points were read after an additional 4 hr at 4 C. Control serum from MCMV hyperimmunized adult mice was included in each set of assays and regularly yielded titers of 1:128.

RESULTS

Attenuation of MCMV in cell cultures. Sequentially passaged MCMV pools were assayed in MECC. Whereas a slight drop in titer occurred on the first and second passage through MECC, subsequent fluids harvested when monolayers showed a 50 to 75% cytopathogenic effect varied only slightly from a titer of 10^6 PFU/ml. There was no discernable difference in plaque size or morphology between wild and passaged material.

Lethality for sucklings. Fig. 1A shows the cumulative mortality induced by 800 PFU of wild, third, or fifth passage MCMV when given ip to groups of 25 to 30 (48-hr-old) suckling mice. Deaths during the first 72 hr after inoculation were disregarded because of the undefinable role of cannibalism in that interval after handling: no difference in overall results would have been seen had these data been included. As expected from previous studies, this relatively high dose of $MCMV_0$ induced death of nearly all inoculated animals within the first week of infection. The absence of lethal effects of $MCMV_3$ and $MCMV_5$ for sucklings during this interval was remarkable. Those animals that did succumb to the attenuated virus died much later in the course of the infection.

Fig. 1B shows the result of using 80 PFU of wild, first, third, or fifth passage virus as inoculum. The virus used in this experiment represented a series of passages from a different wild pool. Each group of animals contained 35 to 45 sucklings inoculated ip at 48 to 72 hr of age. Animals given $MCMV_0$ again sustained a high mortality rate although deaths occurred somewhat later than those seen with the higher dose of virus. Mice inoculated with first passage MCMV had a relatively low early mortality rate, but they suffered a nearly universal failure to thrive, could not be weaned until 4 weeks of age, and deaths of members of this group of animals continued to occur for several weeks. By contrast, $MCMV_3$ and $MCMV_5$ recipients appeared healthy and grew as well as control litters. Recipients of $MCMV_5$ began to die toward the end of the suckling period. These animals differed from the $MCMV_1$ group in that they appeared

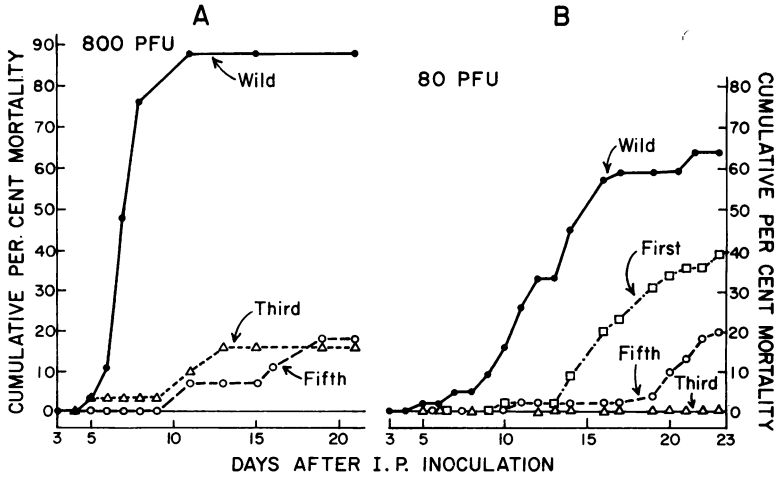

FIG. 1. *Cumulative per cent mortality of mice inoculated at 48 to 72 hr of age with wild or attenuated MCMV. Deaths in first 3 days were not included in results. (A) Each group includes 25 to 30 sucklings inoculated ip with 800 PFU on day zero. (B) Each group includes 35 to 45 sucklings inoculated ip with 80 PFU on day zero. Control litters, 50 sucklings in each experiment, were inoculated with an equal volume of diluent on day zero. They suffered no mortality after the first 72 hr.*

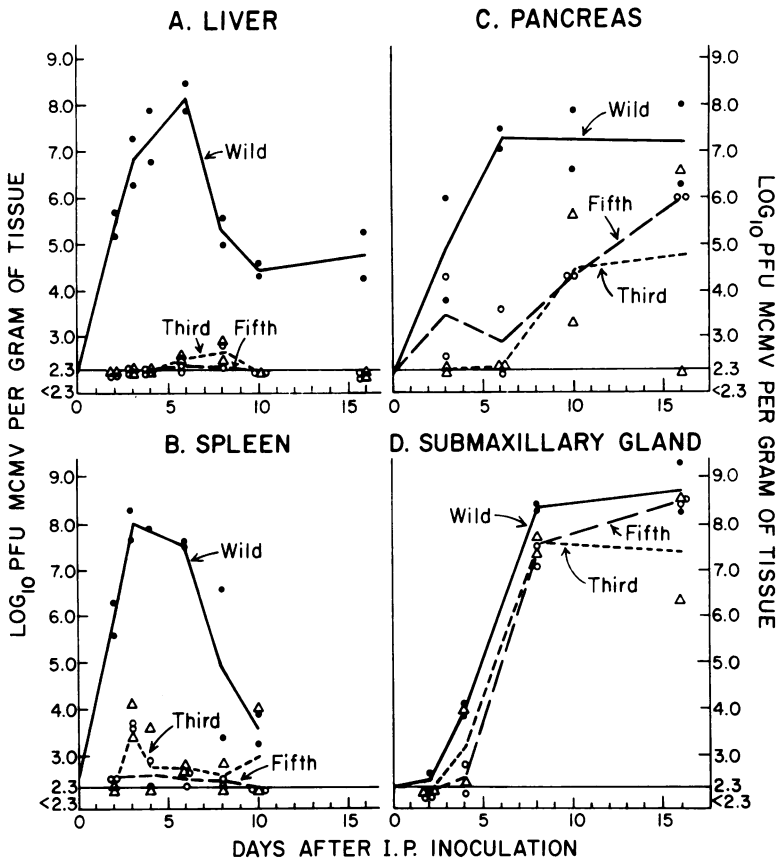

FIG. 2. *Concentration of MCMV in tissues of 3-week-old mice inoculated ip on day zero with $10^{5.0}$ PFU of $MCMV_0$, $MCMV_3$, or $MCMV_5$. All tissue extracts were assayed by centrifugal inoculation to identify maximum quantities of infectious virus. Curves represent geometric mean values of the plotted points. Each point represents the viral titer in pooled organs from 2 to 3 mice sacrificed at the designated interval after injection.*

healthy until a few days before death and then became cachectic and lethargic.

Capacity of attenuated MCMV to multiply in various organs of weanling mice. The marked alteration of lethality for suckling mice raised the possibility that passaged MCMV might multiply poorly if at all in older mice. Thus, a study was designed to compare multiplication of wild and attenuated virus in liver, spleen, submaxillary gland, and pancreas of mice inoculated ip at 3 weeks of age with $10^{5.0}$ PFU of $MCMV_0$, $MCMV_3$, or $MCMV_5$.

Fig. 2 shows the results of these assays. As noted in previous studies (9, 12), the liver and spleen were the sites of vigorous viral multiplication of the wild virus during the first week of infection, whereas submaxillary gland viral titer remained low for several days and then rose to high levels which were maintained thereafter. Pancreatic infection followed a course different from both these patterns. Multiplication began very early, but as in the case of the submaxillary gland the viral titers then remained high throughout the interval studied.

$MCMV_3$ and $MCMV_5$ were very similar in the patterns of infection they produced. Both groups demonstrated nearly complete absence of virus in the liver in spite of the fact that the ip route of inoculation had been used. Spleen titers rose briefly and then fell to undetectable levels by the sixth day. In sharp contrast, passaged MCMV thrived in the submaxillary glands. The outcome of pancreatic infection with attenuated virus was variable. There was a definite delay in onset of multiplication, and one pair of animals sacrificed 16 days after inoculation with $MCMV_5$ had no detectable virus in the pancreas. The remaining pairs assayed were undergoing chronic pancreatic infection.

Antibody responses of mice infected with wild or attenuated MCMV. Fig. 3A and 3B show the hemagglutinin (HA) and hemolysin (HL) titers,

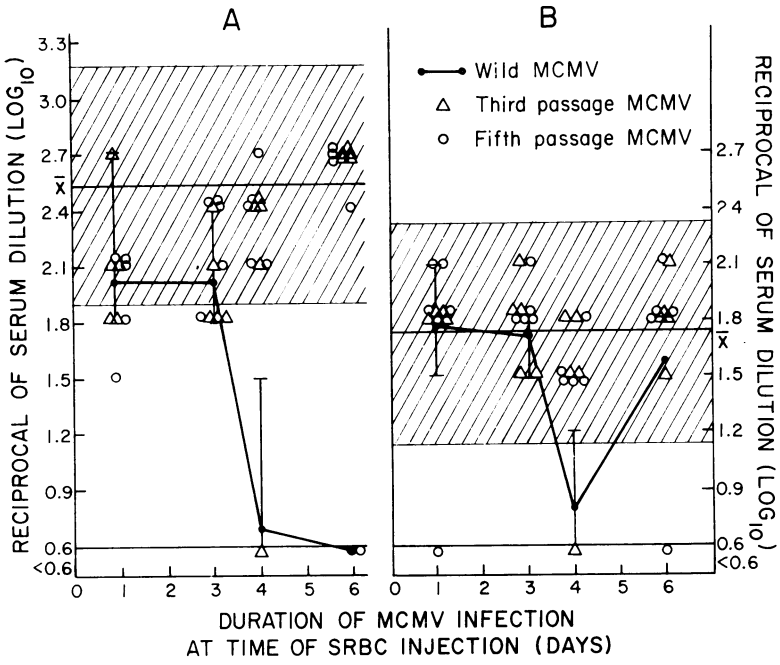

FIG. 3. *Serum hemagglutinin (A) and hemolysin (B) responses of various groups of mice infected with $10^{5.0}$ PFU of $MCMV_0$, $MCMV_3$, or $MCMV_5$ on day zero. Mice were inoculated with 2 to 4 $\times$ 10^8 SRBC on day 1, 3, 4, or 6 as indicated and bled 7 days later. In each figure shaded areas represent 2 SD about the mean ($\bar{x}$) of 20 sera from uninfected animals given the same dose of SRBC. Means and ranges of values for $MCMV_0$-infected mice represent the values for 10 animals at each point. Individual values are plotted to show HA and HL titers achieved by mice infected with attenuated MCMV and given SRBC at comparable times. No range is depicted for HL titers in $MCMV_0$-infected animals on day 6 since within the group of 10 mice values varied from <0.6 to 2.7.*

respectively, achieved by individual mice 7 days after ip injection of 2 to 4 $\times$ 10^8 SRBC. The interval between inoculation with wild or attenuated MCMV and subsequent injection of SRBC antigen is represented on the ordinate in each case. The mean and range of values of 7-day serum antibody responses of 10 $MCMV_0$-infected animals are shown at each time of testing, and the results (Fig. 3A) confirm earlier findings of nearly complete HA unresponsiveness when SRBC are given on day 4 or 6 of wild virus infection (8). In general HA response was more profoundly and generally suppressed over a longer interval with $MCMV_0$ than was HL antibody, and in addition there was a striking lack of correlation between HA and HL responses of individual animals. Mice showing total suppression of HA response had high concentrations of HL antibody in several instances. This is consistent with the suggestion that HL and HA responses to SRBC represent the function of entirely separate groups of cells (13).

Data for mice infected with attentuated MCMV are plotted individually to show that with few exceptions these animals showed no evidence of profound immunosuppression, even when antigen was given on the 4th or 6th day of infection. However, in several cases individual animals infected with $MCMV_3$ or $MCMV_5$ failed to respond to SRBC with either HA or HL antibody.

Since the large majority of animals infected with attenuated virus were normally responsive to SRBC antigen, the possibility arose that the relative delay in appearance of MCMV neutralizing antibody previously described after wild virus infection (18) might also have been affected by tissue culture passage. Thus sera from two pools of three mice each were assayed for both neutralizing and CF antibody at 4, 11, and 13 days after inoculation of $10^{5.0}$ PFU of $MCMV_0$, $MCMV_3$, or $MCMV_5$. No neutralizing or CF antibody rise was observed until the 13th day when barely detectable titers (1:8 or 1:16) of both types of antibody were found in some mice in each group. Thus, attenuation by tissue culture passage did not alter the relatively poor, early, serologic response of infected mice to MCMV antigens.

Interferon responsiveness of mice infected with attenuated MCMV. During the first 2 weeks of

infection, $MCMV_0$ induces an inability of the host to respond with circulating interferon to intravenous administration of NDV (9). Attenuation deleted this aspect of acute MCMV infection (Table 1).

Ability of antiserum prepared with $MCMV_0$ to neutralize attenuated virus. To test the possibility that antigenic alteration might be associated with attenuation, a comparison was made of the neutralizing capacity of rabbit anti-MCMV serum prepared against $MCMV_0$ when tested against 120 to 160 PFU (centrifugal adsorption) of either $MCMV_0$, $MCMV_3$, or $MCMV_5$. Simultaneous plaque-reduction neutralization assays yielded nearly identical results with each of the challenge viruses, supporting the hypothesis that the attenuated agents were very closely related to wild MCMV.

Protection of survivors of neonatal infection with attenuated virus. To further explore the possibility of antigenic change, survivors of neonatal inoculation with $MCMV_3$, $MCMV_5$, or controls from the experiment represented in Fig. 1B were tested for their ability to withstand challenge at 5 weeks of age with a potentially lethal dose ($10^{6.2}$ PFU) of $MCMV_0$. Sixteen of 18 mice given only diluent in the neonatal period died within 1 week after $MCMV_0$ challenge, whereas groups of 15 mice previously infected with $MCMV_3$ or $MCMV_5$ remained completely healthy. Thus, complete protection of the survivors of attenuated MCMV neonatal infection was in marked contrast to the susceptibility of mice that had not been previously infected with attenuated virus.

Studies of mechanism of attenuation. Simple mutation or deletion of the virulent portion of the MCMV population might explain the rapid attenuation seen. Were this the sole mechanism, back passage of attenuated virus through mice would be unlikely to restore virulence rapidly or regularly. To study the effect of back passage in mice, multiplication in the liver was chosen as a test for return of virulence. Mice aged 3 to 4 weeks were inoculated ip with $10^{5.4}$ PFU of MCMV obtained from submaxillary glands of animals after 2 weeks of infection with $MCMV_0$, $MCMV_3$, or $MCMV_5$. The livers of three pairs in each virus group were harvested and assayed for MCMV on day 5 of the ensuing infection. The MCMV found in these livers represented the progeny of the second back passage of attenuated virus in mice (and of continuous mouse passage in the case of $MCMV_0$). A third back passage was done in the same manner with submaxillary glands harvested at 2 weeks from other recipients of the second back passage virus. Back passage in mice quickly restored virulence in terms of liver multiplication (Table 2).

Since wild pools of MCMV, passaged continuously in mice, might contain infectious agents in addition to MCMV or might contain discrete virulent and avirulent populations, studies were undertaken with six plaque-purified clones derived from wild MCMV pools to test for variable pathogenicity. To test each clone, litters of 48-hr-old mice were inoculated ip with 1,000 PFU per mouse. Two litters inoculated with 1,000 PFU of wild MCMV sustained 100% mortality by the 11th day postinoculation,

TABLE 1. *Serum interferon response of groups of mice infected 4 days previously with wild or attenuated murine cytomegalovirus (MCMV)*

Initial inoculum (PFU ip)[a]	Serum interferon level[b] 5 hr after iv inoculations of NDV[c] ($10^{8.0}$PFU)
Saline	3,000, 1,200
$MCMV_0$ ($10^{5.0}$)	<30, <30
$MCMV_3$ ($10^{5.0}$)	3,000, 1,600
$MCMV_5$ ($10^{5.0}$)	1,200, 1,000

[a] Abbreviations: PFU, plaque-forming units; ip, intraperitoneally.

[b] Interferon titers, representing pools of sera from three mice for each determination, are expressed as the reciprocal of that serum dilution which caused a 50% reduction in 40 PFU encephalomyocarditis virus after 3-hr pretreatment of L cells.

[c] Abbreviations: iv, intravenous NDV, Newcastle disease virus.

TABLE 2. *Reacquisition of murine cytomegalovirus (MCMV) virulence for mouse liver on back passage in mice*

Virus	Second passage in mice		Third passage in mice	
	PFU/g of liver	Mean	PFU/g of liver	Mean
$MCMV_0$	6×10^{7}[a]	1.3×10^{6}	2×10^{7}	1.3×10^{6}
$MCMV_0$	1×10^{6}		1×10^{6}	
$MCMV_0$	7×10^{4}		1×10^{5}	
$MCMV_3$	7×10^{7}	5×10^{6}	2×10^{6}	8×10^{5}
$MCMV_3$	2×10^{7}		1×10^{6}	
$MCMV_3$	6×10^{4}		4×10^{5}	
$MCMV_5$	7×10^{4}	1.6×10^{4}	4×10^{5}	2×10^{5}
$MCMV_5$	1×10^{4}		2×10^{5}	
$MCMV_5$	7×10^{3}		9×10^{4}	

[a] Each figure represents PFU of MCMV in a pair of livers harvested 5 days after inoculation with 3×10^{5} PFU (submaxillary gland extract) of the preceding mouse passage.

whereas cumulative mortality data for recipients of cloned virus, which had been passaged four times in MECC during purification, were almost exactly analogous to the results of attenuated virus infection (Fig. 1A).

Three clones were further compared with wild virus by testing for liver multiplication in weanling mice. Animals were inoculated with 10^5 PFU of wild or plaque-purified virus. On day 5 livers of three pairs of animals in each group were harvested and assayed by centrifugal adsorption. Titers of MCMV/g of liver ranged from <200 to 8,000 in nine groups that had received cloned MCMV, whereas wild virus multiplied to levels of 5 to 660 $\times$ 10^4 in three pairs of mice. Submaxillary glands from recipients of these clones were harvested on day 12 of infection, and 10^5 PFU of virus from these glands were inoculated ip into weanling mice. One passage in mice restored to plaque-purified cloned MCMV its capacity for vigorous reticuloendothelium multiplication as demonstrated by equally high liver titers on day 5 of infection compared with wild virus recipients.

Thus, several clones of MCMV passaged in tissue culture during purification had the same properties of diminished virulence as did routinely attenuated pools and rapidly regained full virulence as measured by liver multiplication on back passage in mice.

DISCUSSION

Passage of virulent viruses in tissue culture frequently results in reduction of pathogenicity for the natural host. Such loss of virulence usually occurs gradually over many passages, and the derived agents often display antigenic changes and increased adaptation to tissue culture, such as altered or accelerated development of cytopathogenicity. Other examples of attenuation for animal hosts in which more rapid change in virulence occur generally represent the emergence of stable mutants or, similarly, can be achieved by identification and clonal selection of plaque variants from a heterogeneous wild virus population.

There have been few studies of attenuation of herpesviruses for animal hosts. Hinze and Walker (4) described one such system in which chronically infected herpes simplex carrier cultures, requiring constant presence of antibody for their maintenance, gradually (after 6 months) yielded a herpesvirus with altered cytopathogenicity in cell culture and markedly reduced neurovirulence for mice by intracranial inoculation. These changes were stable so long as antibody remained in the extracellular environment of the cultures. Wheeler (14) achieved a gradual change in herpes simplex tissue culture behavior and virulence for mice by similar means using antibody during induction of changes in culture. The resultant virus differed in that once the change in pathogenicity had occurred, it was stable and no longer required antibody. Marek's disease agent is generally accepted to be a herpesvirus and has recently been found to resemble the B subgroup of herpesviruses of which most members are cytomegaloviruses (5). One of the most detailed studies of herpesvirus attenuation reported to date deals with the gradual loss of pathogenicity of Marek's disease agent for chickens over 33 passages in chick embryo cell culture (1). Prior infection with the attenuated strain protects chickens against oncogenic effects of the virulent agent (2). Attenuation in this system is thought to result from steady selective pressure in the tissue culture environment favoring avirulent macroplaque-forming components of the wild pool and finally deleting the virulent microplaque formers entirely so that the pool irreversibly loses its pathogenic component for chickens.

The attenuation of MCMV is unique in the combination of factors it represents. It occurs with remarkable rapidity without associated changes in neutralizing or protective antigens, or both, and without obvious changes in rate or characteristics of tissue culture cytopathology, and it is rapidly reversible upon back passage in mice.

Several features of the infection with attenuated virus deserve note. The overwhelming mortality rate seen in suckling mice during the first week of infection with wild virus was deleted entirely even after one passage in tissue culture. However, the attenuation was not complete, for there was appreciable mortality, or failure to thrive among surviving recipients of attenuated MCMV, or both throughout the period of observation. The disease observed in these animals is unexplained but may correlate with pancreatic infection, for in older animals spleen and liver multiplication was virtually eliminated by tissue culture passage, and the course of submaxillary gland infection was unchanged, whereas chronic, high titer pancreatic infection was definitely delayed but not eliminated.

In view of the vigorous multiplication of attenuated virus in submaxillary and pancreatic glandular tissue, the failure of effective multiplication in liver and spleen appears to represent a differential alteration in virulence for a given tissue. In this context it seems unlikely that failure of access or adsorption could explain the change in behavior of attenuated virus, for all

inocula were given by the ip route, and receptor sites of organs other than liver and spleen were apparently adequate for initiation of infection. An alternative explanation of the failure to replicate would be that host mechanisms for cytoplasmic degradation of viral progeny were in some way more efficacious against tissue culture derived inocula. It has been suggested previously that MCMV infection of mouse liver and spleen is terminated by lysosomes functioning as virus "graveyards" for virions once they leave the nuclei of infected cells (12). The fact that all MCMV variants readily infect submaxillary glands is consistent with this hypothesis, since it is thought that high titer chronic infection of this organ results from an incapacity to inactivate complete virions once they leave the nucleus (12). Further exploration of these hypotheses is planned.

The correlation between attenuation and loss of immunosuppressive properties strengthens the supposition that the initial involvement of reticuloendothelial organs in some way mediates the transient immunosuppressive effects of MCMV (8). It has been suggested that MCMV induces in the host a homograft-like response by virtue of alteration of cytoplasmic membranes of infected reticuloendothelial cells (3). Recent studies in this laboratory (*unpublished data*) suggest that virulent MCMV significantly alters nonspecific cell-mediated immune responses when these are tested at a time of maximum immunosuppression. This combination of events would be consistent with a homograft response, and were these mechanisms involved in virulent infection the failure of attenuated virus to establish infection in the reticuloendothelial system would be sufficient explanation of the abolition of immunosuppressive effects. The occasional animal which appeared to be immunosuppressed after attenuated MCMV infection is consistent with the hypothesis that attenuation is host-mediated, since individual outbred mice would be expected to vary in the efficacy of their defense against such infection and failure of an individual animal to limit MCMV multiplication would then result in immunosuppression.

Mechanisms by which tissue culture environment might induce rapid attenuation of MCMV are difficult to postulate, but several observations are pertinent. First, the rapid reversibility of the changes studied makes it highly unlikely that a simple mutation could be the basis of such events. Secondly, the hypothesis must be entertained that the heterogeneity of the initial population represents a mixture of two discrete viruses. But plaque purification as well as neutralization and protection studies show that no important antigenic deletions had occurred with passage to explain the altered pathogenetic potential. In spite of these protection studies, the possibility still remains that a ubiquitous murine virus, for which MECC is nonpermissive, is a necessary cofactor for full expression of MCMV virulence. Such an explanation of the rapid return of virulence would require the regular occurrence of the unknown agent in the mice studied. A number of agents of mice such as murine leukemia, LDH, or mouse hepatitis viruses could fulfill these requirements. The possibility that contaminating virus is present in pools of mouse-passaged MCMV is being explored.

Remaining acceptable hypotheses include the possibility that there are, in wild MCMV pools, virions that are heterogeneous in their ability to replicate in cells of reticuloendothelial organs or that tissue culture passage alters the ability of MCMV progeny to resist cytoplasmic destruction in cells where these inactivating (possibly lysosomal) mechanisms are operative. The latter hypothesis is attractive in that submaxillary gland, which appears to lack such cytoplasmic terminating mechanisms in wild MCMV infection (12), continues to manifest a marked susceptibility to MCMV after tissue culture attenuation. Those features of the tissue culture environment that affect such a rapid change await further study.

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